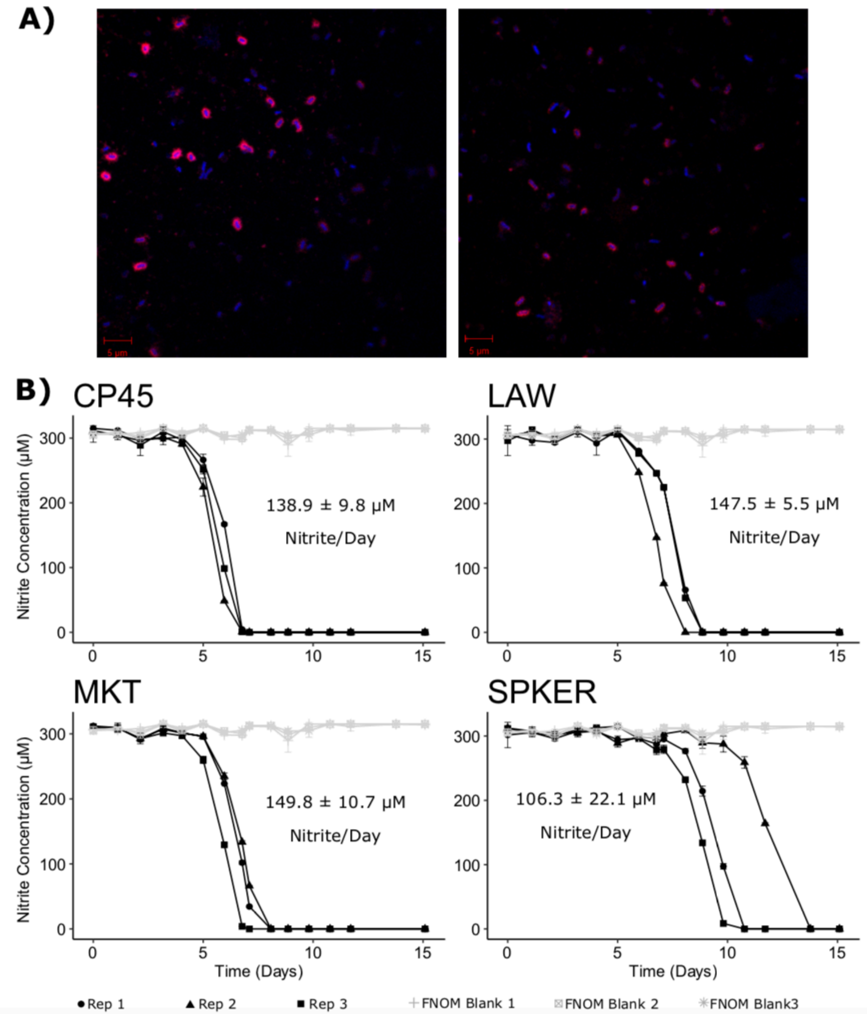
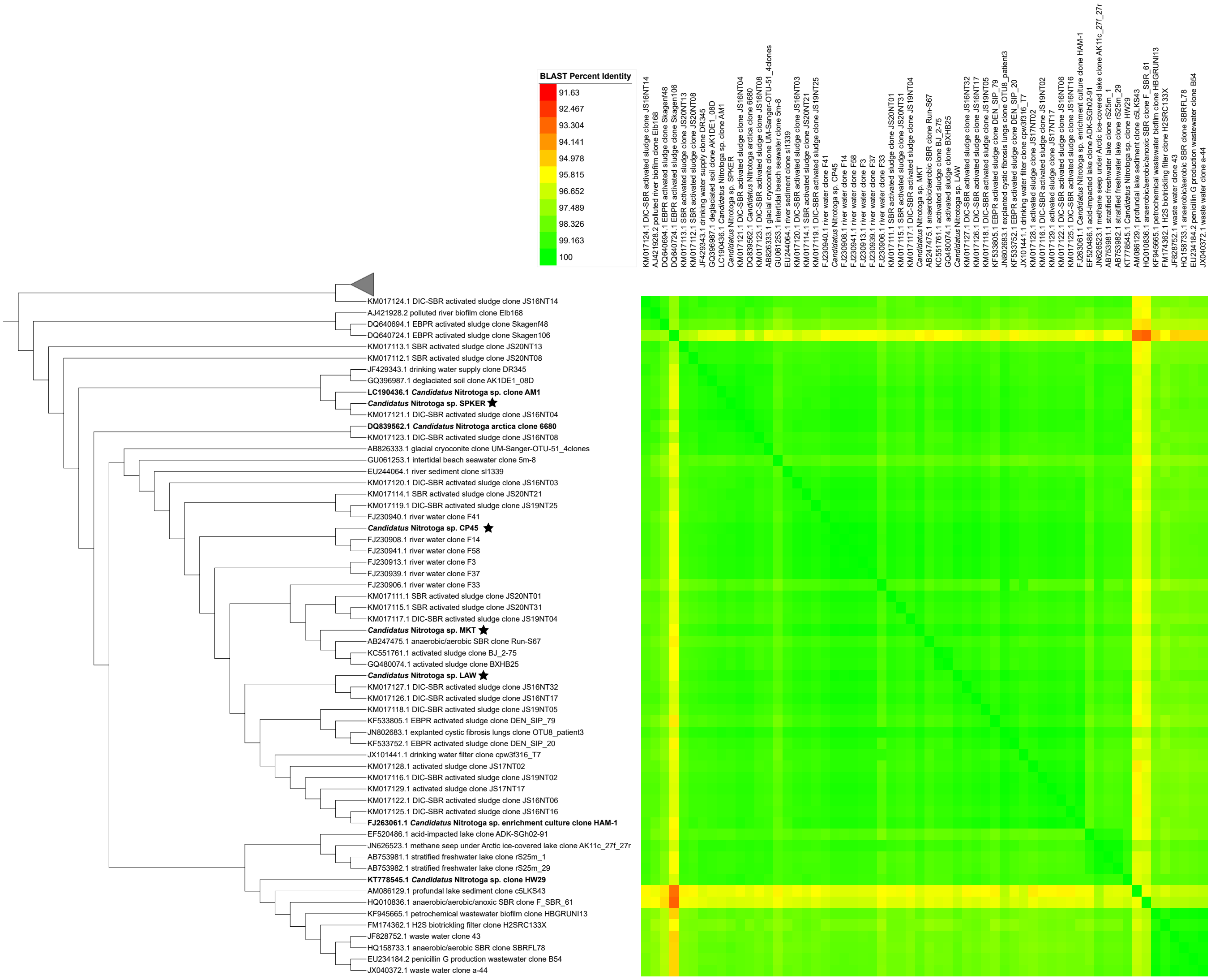


Supplemental Figure S1. Nitrite-oxidizing enrichment cultures showed A) hybridization with a *Ca.* Nitrotoga-specific fluorescent probe (Ntoga122) and B) consumption of nitrite over time. Representative FISH images from CP45 show Ntoga122-hybridized cells in red and all cells stained with DAPI in blue. Cells have coccoid to short rod-shaped morphology. Ring-like staining may be associated with incomplete permeabilization or a ribosome-free region of the cytoplasm, such as the large intracellular inclusions seen in other *Ca.* Nitrotoga cells. For nitrite consumption measurements, each enrichment culture was inoculated in three replicates, and nitrite concentration was quantified colorimetrically in triplicate at each time point. Error bars show the standard deviation of each time point; error bars that appear to be missing are too small to be visualized. Sterile FNOM was used as a control and plotted with each culture. Logarithmic declines in nitrite concentration were used to calculate the nitrite oxidation rate for each biological replicate. The average nitrite consumption per day is shown with standard deviation among triplicate cultures.



Supplemental Figure S2. Pairwise BLAST comparisons of 60 *Ca.*Nitrotoga and *Ca.*Nitrotoga-like 16S rRNA gene sequences ≥1,300 bp in length presented as a heatmap. The phylogeny from Figure 2 is displayed on the left with branch lengths ignored. Bolded sequence names have been enriched in culture. Nodes with a star represent organisms presented in this study.

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               Ca. Nitrotoga sp. CP45 NxrB   1 MAKVRNWQLGREMDYPYEENRPGRQVSMLFDLNKCIACQSCTMACKTTWTAGKGQ 55
                Ca. Nitrotoga sp. LAW NxrB   1 MAKVRNWQLGREMDYPYEENRPGRQITMLFDLNKCIACQSCTMACKTTWTAGKGQ 55
                Ca. Nitrotoga sp. MKT NxrB   1 MAKVRNWQLGREMDYPYEENRPGRQITMLFDLNKCIACQSCTMACKTTWTAGKGQ 55
              Ca. Nitrotoga sp. SPKER NxrB   1 MAKVRNWQLGREMDYPYEENRPGRQVSMLFDLNKCIACQSCTMACKTTWTAGKGQ 55
      (CBK42947.1) Nitrospira defluvii NxrB   1 MPEVYNWQLGRKMLYPYEERHPKWQFAFVFNINRCLACQTCSMADKSTWLFSKGQ 55
(WP_053381688.1) Nitrospira moscoviensis NxrB   1 MPEVYNWQLGRKMLYPYEERHPKWQFAFVFNINRCLACQTCSMADKSTWLFSKGQ 55
    (WP_080885590.1) Nitrospira japonica NxrB   1 MPEVYNWQLGRKMLYPYEERHPKWQFAFVFNINRCLACQTCSMADKSTWLFSKGQ 55
      (AGF29470.1) Nitrospina gracilis NxrB   1 MPEVYNWQLGRMMTYVYEEKHPKEQFTFVFNTNRCIACQTCTMAHKSTWTFSKGQ 55
(KMP11422.1) Ca. Nitromaritima SCGC_AAA799_A02 NxrB   1 MPEVYNWQLGRMMTYVYDEKHPKEQFTFVFNTNRCIACQTCTMAHKSTWTFSKGQ 55
   (AAR92470.1) Nitrobacter winogradskyi NxrB   1 -------------------MDIRAQVSMVFHLDKCIGCHTCSIACKNIWTDRKGQ 55
    (WP_005004545.1) Nitrococcus mobilis NxrB   1 -------------------MDIRAQVSMVFHLDKCIGCHTCSIACKNIWTDRQGT 55
     (CCF85658.1) Nitrolancea hollandica NxrB   1 -------------------MDIRAQVSMTFHLDKCIGCHTCSIACKNIWTDRKGT 55
          (CRI68047.1) Thiocapsa sp. KS1 NxrB   1 -------------------MRIKQQMGIVFNLDKCLGCQTCTIACKNVWTNREGA 55

               Ca. Nitrotoga sp. CP45 NxrB 206 KNWGFFFPRICNHCTFPGCLAACPRKAIYKRQEDGIVLIDASRCRGYRECVAACP 260
                Ca. Nitrotoga sp. LAW NxrB 206 KNWGFFFPRICNHCTFPGCLAACPRKAIYKRQEDGIVLIDASRCRGYRECVAACP 260
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              Ca. Nitrotoga sp. SPKER NxrB 206 KNWGFFFPRICNHCTFPGCLAACPRKAIYKRQEDGIVLIDASRCRGYRECVAACP 260
      (CBK42947.1) Nitrospira defluvii NxrB 206 ETYFFYLQRICNHCTYPGCLAACPRKAIYKRPEDGIVLIDQNRCRGYKKCVEQCP 260
(WP_053381688.1) Nitrospira moscoviensis NxrB 206 ETFFFYLQRICNHCTYPGCLAACPRKAIYKRPEDGIVLIDQNRCRGYKKCVEQCP 260
    (WP_080885590.1) Nitrospira japonica NxrB 206 ETFFFYLQRICNHCTYPGCLAACPRKAIYKRPEDGIVLIDQNRCRGYKKCVEQCP 260
      (AGF29470.1) Nitrospina gracilis NxrB 206 KIWFFYLQRICNHCTYPGCLAACPRKAIYKRQEDGIVLIDQSRCRGYKKCVEQCP 260
(KMP11422.1) Ca. Nitromaritima SCGC_AAA799_A02 NxrB 206 KIWFFYLQRICNHCTYPGCLAACPRKAIYKRQEDGIVLIDQSRCRGYKKCVEQCP 260
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          (CRI68047.1) Thiocapsa sp. KS1 NxrB 206 NSFMMYLPRICNHCLNPACVGSCPSGANYKREEDGVVLIDQDRCRGWRYCVSGCP 260

               Ca. Nitrotoga sp. CP45 NxrB 261 YKKSFYNDTTRTGEKCISCYPKVEAG--------LMTQCVTQCIGKIRLFGFKSA 315
                Ca. Nitrotoga sp. LAW NxrB 261 YKKSFYNDTTRTGEKCISCYPKVEAG--------LMTQCVTQCIGKIRLNGFKSA 315
                Ca. Nitrotoga sp. MKT NxrB 261 YKKSFYNDTTRTGEKCISCYPKIEAG--------LMTQCTTQCIGKIRLNGFKSA 315
              Ca. Nitrotoga sp. SPKER NxrB 261 YKKSFYNDTTRTGEKCISCYPKVEAG--------LMTQCVTQCIGKIRLFGFKSA 315
      (CBK42947.1) Nitrospira defluvii NxrB 261 FKKPMYRGTTRVSEKCIACYPRIEGKDPLTGGEPMETRCMAACVGKIRMQSLMRI 315
(WP_053381688.1) Nitrospira moscoviensis NxrB 261 FKKPMYRGTTRVSEKCIACYPRIEGKDPLTGGEPMETRCMAACVGKIRMQSLVRI 315
    (WP_080885590.1) Nitrospira japonica NxrB 261 FKKPMYRGTTRVSEKCIACYPRIEGKDPLTGGEPMETRCMAACVGKIRMQSLVRI 315
      (AGF29470.1) Nitrospina gracilis NxrB 261 YKKPMFRGTTRISEKCIACYPRIEGLDPLTEGDQMETRCMAACVGKIRLQGLVKI 315
(KMP11422.1) Ca. Nitromaritima SCGC_AAA799_A02 NxrB 261 YKKPMFRGTTRISEKCIACYPRIEGLDPLTEGDQMETRCMAACVGKIRLQGLVKV 315
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          (CRI68047.1) Thiocapsa sp. KS1 NxrB 261 YKKTYYNWRTGKSEKCILCFPRLETG--------QPPMCFQACVGRIRYLGPLLY 315
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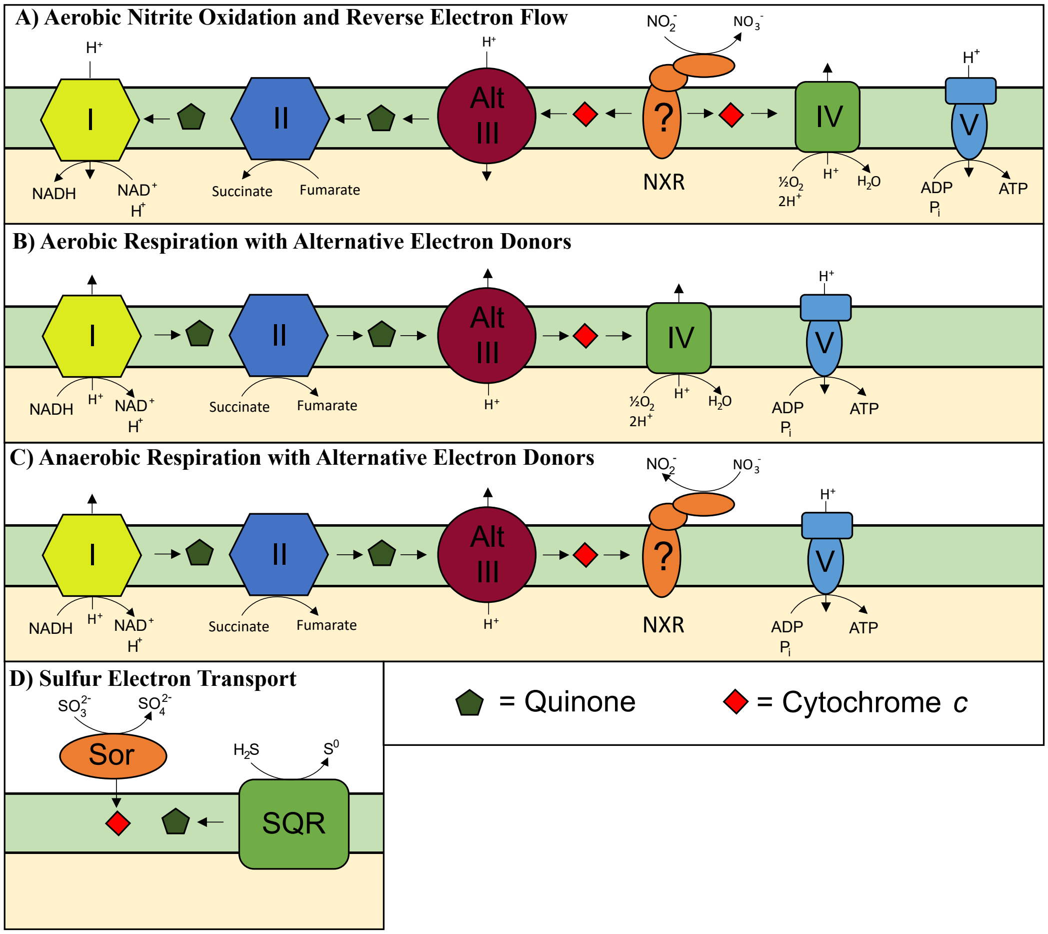
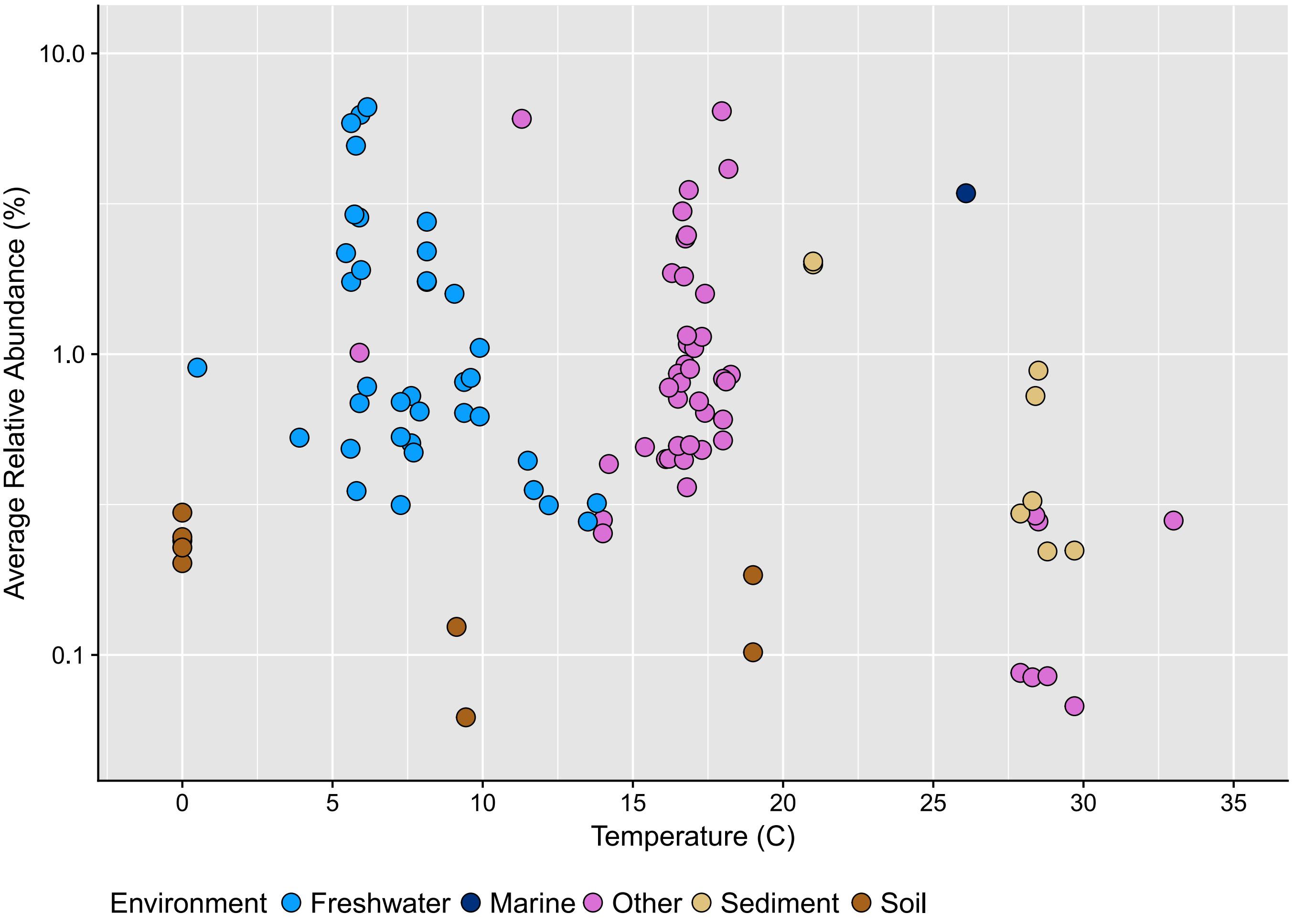
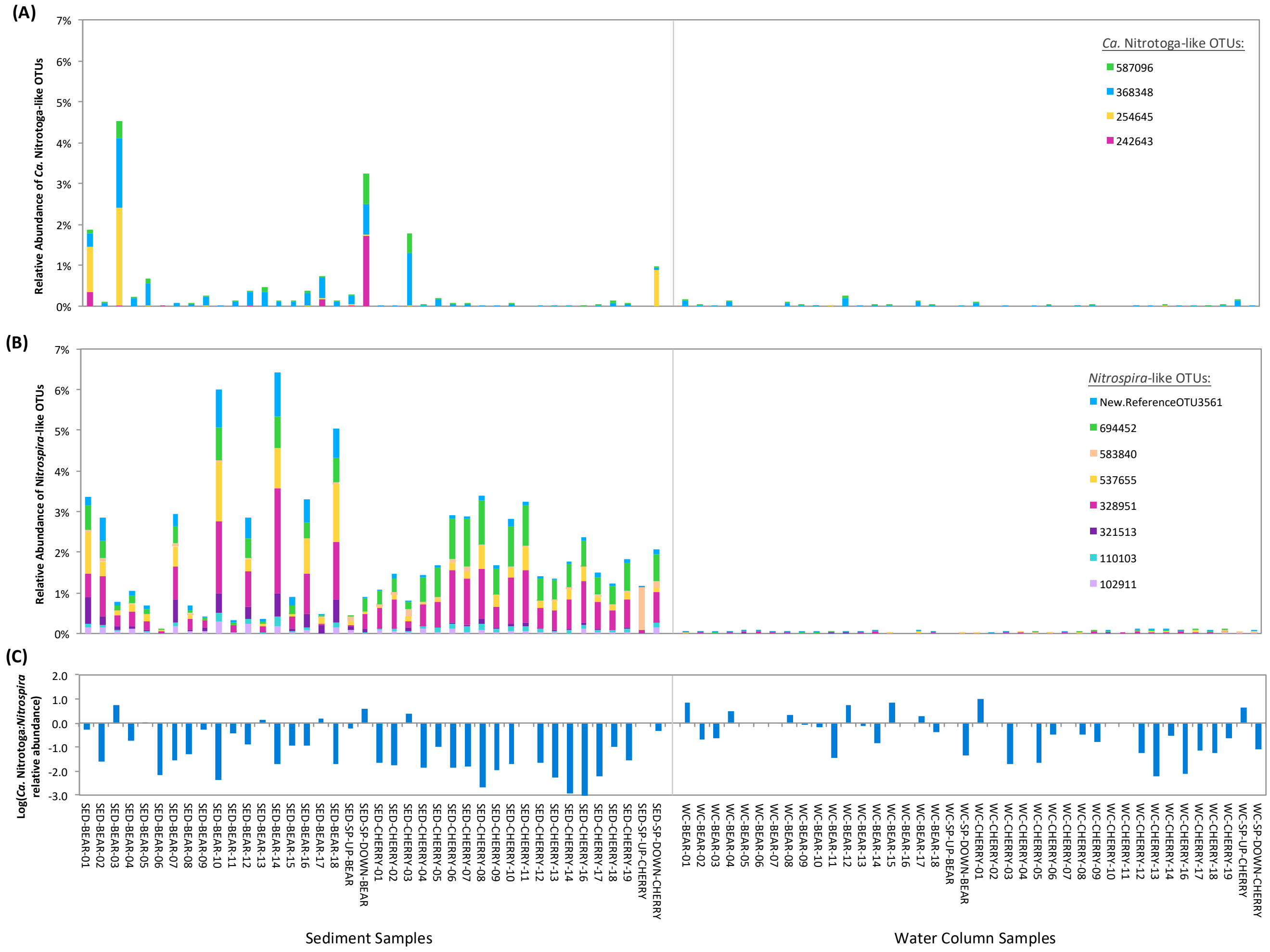
Supplemental Figure S3. Partial alignment of important residues from selected NxrB subunits representing the *Ca.* Nitrotoga (Red), as well as canonical periplasmic-facing (Purple) and cytoplasmic-facing (Blue/Green) NXR. Highlights represent Fe-S cluster binding residues (Yellow).



Supplemental Figure S4. Schematic of electron transfer in *Ca.* Nitrotoga based on genomic evidence. **A)** Canonical nitrite oxidation performed by NXR will liberate two electrons onto cytochrome *c* and flow forwards to the terminal oxidase (Complex IV), or backwards to regenerate NADH via Alternative Complex III and the quinone pool, or to generate biochemical intermediates via Complex II. The question mark located in the NxrC subunit symbolizes the uncertainty in whether or not the holoenzyme is anchored to the cell membrane. **B)** Aerobic respiration with alternative electron donors such as NADH derived from organic carbon utilization. **C)** Hypothesized anaerobic respiration with alternative electron donors (e.g., NADH) with the reduction of nitrate to nitrite via NXR as seen for other NOB. **D)** Electrons derived from reduced sulfur compounds (sulfites or sulfides) are transferred to cytochrome *c* or quinone, which can enter at any point in electron transfer shown in panels B or C.



Supplemental Figure S5. Analysis of *Ca.* Nitrotoga-like OTUs from public 16S rRNA gene amplicon studies (deposited as SRA runs; clustered at ≥97% identity by IMNGS) with available environmental temperature metadata. Temperature data was extracted from 101 unique BioSamples from all SRA runs with *Ca.* Nitrotoga-like OTUs, and plotted against the average relative abundance of *Ca.* Nitrotoga-like OTUs. Average relative abundance was calculated across the four queried *Ca.* Nitrotoga 16S rRNA gene sequences after instances with <100 reads were removed.



Supplemental Figure S6. Relative abundance of **A)** *Ca.* Nitrotoga- and **B)** *Nitrospira*-like 16S rRNA gene sequence OTUs from water column ("WC") and sediment ("SED") samples in Bear Creek ("BEAR"), Cherry Creek ("CHERRY"), and the upstream ("SP-UP") and downstream ("SP-DOWN") sites at their respective confluences with the South Platte River. OTUs were based on the amplification with general 16S rRNA gene primers targeting the total bacterial community and were grouped at 97% nucleotide identity. *Ca.* Nitrotoga and *Nitrospira* OTUs were identified based on BLAST searches against the SILVA rRNA gene database. **C)** Log ratio of the summed relative abundance of *Ca.* Nitrotoga- to *Nitrospira*-like 16S rRNA gene sequences.